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Effects of Soil Storage on the Microbial Community and Degradation of Metsulfuron-methyl

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The effect storage had on the microbial biomass in two soils (Trevino and Fargo) was compared to the effect storage had on each soil's capacity to degrade metsulfuron-methyl. Soils were collected from the field and used fresh (<3 weeks old) or stored at 20 and 4 °C for 3 or 6 months. The phospholipid fatty acid content of the soils was used to monitor changes in the microbial biomass during storage and incubation in a flow-through apparatus. In both soils, [*phenyl*-U-^{14}C]metsulfuron-methyl was used to monitor changes in the route and rate of degradation along with $^{14}CO_2$ evolution (mineralization). Total microbial biomasses in both soils were significantly reduced for soils incubated in the flow-through apparatus, whereas only the Trevino soil's microbial biomass was significantly reduced as a result of storage. The microbial communities of both soils were significantly different as a result of storage as shown by discriminant analysis. In both soils, degradation rate, pathway of degradation, and mineralization of metsulfuron-methyl were significantly affected by storage compared to fresh soil. The half-life of metsulfuron-methyl increased significantly ($P < 0.05$) in the Trevino soil from 45 days (fresh) to 63 days (stored soil), whereas in the Fargo soil half-lives increased significantly ($P < 0.05$) from 23 days (fresh) to 29 days (soils stored for 6 months). In both soils, mineralization of [^{14}C]metsulfuron-methyl was significantly ($P < 0.05$) higher in fresh soils compared to stored soils. The degradation pathways of metsulfuron-methyl changed with storage as evidenced by the loss of formation of one biologically derived metabolite (degradate) in stored soils compared to fresh soils.

KEYWORDS: Storage; biomass; microbial community; pesticide; metsulfuron-methyl; half-life; degradation pathway

INTRODUCTION

Soils are the primary medium through which most crop protection products (CPP) enter the environment. Regulators are interested in the metabolism and rate of degradation of these compounds in soil in order to evaluate the fate of these compounds in the environment. The registration of new and existing CPP in the United States (*1*) and the European Union (EU) (*2*) requires laboratory soil metabolism studies to be conducted following certain regulatory guidelines. These studies are used to determine half-lives, degradation pathways, and significant metabolites of CPP in the soil environment. Terrestrial soil dissipation in the field and terrestrial ecotoxicological studies are in part dictated by the results obtained in laboratory soil metabolism studies. In addition, soil metabolism studies are one of the key studies used in determining which metabolites are to be included in the ecological risk assessment during the registration process (*3*). Consequently, ensuring that a laboratory soil's intrinsic properties are maintained prior to the initiation of a study is vital because loss of a soil functional property may bias results that will eventually be used in a compound's regulatory evaluation.

The methods used in collecting, processing, and storing soil will greatly influence its integrity, and typically the soil microbial community is the most affected by storage. Improper handling of soil that negatively influences the soil microbial community can affect the degradation rates of compounds that are degraded biologically. EU guidelines (*4*) specify that moist soil samples be processed through a 2 mm sieve, collected during nonstressed conditions (i.e., no flooding, drought, or freezing events), and stored for no longer than 3 months at 4 °C. Storage of soil can be extended beyond 3 months by demonstrating the microbial community remained viable (*5*) and

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Table 1. Select Properties of Soils

soil	pH	OC[a]	soil water content at 33 kPa (%)	particle size analysis sand (%)	silt (%)	clay (%)
Trevino	8.0	1.3	20	21	65	14
Fargo	7.7	3.2	40.1	20	56	24

[a] Organic carbon.

if soils are stored frozen (*6*). The guidelines are supported by findings that environmental stress reduces the microbial diversity and functionality in soil (*7*−*9*), whereas the processing of moist soil through a 2 mm sieve has been shown to have little or no impact on the microbial diversity and functionality for cultivated soils (*10*, *11*). However, the scientific evidence supporting storage guidelines is not well documented.

In general, the literature has shown that storage of soil has negative impacts on the soil microbial community, and these include declines in the biomass (*7*, *12*, *13*), community structure (*14*, *15*), genetic diversity (*16*), and functionality (*17*). The literature is ambiguous on the ultimate result of these effects on the rate of degradation of CPP in soil. One study has demonstrated deleterious effects in the degradation of thiocarbamate as a result of soil storage (*7*), whereas two other studies have measured little or no change in the degradation of other CPP following storage (*18*, *19*). These studies used the loss of parent compound or mineralization of a radiolabeled compound as a benchmark for their evaluation of the effects storage has on the degradation of CPP. However, considering that the purpose of soil metabolism studies is not only to establish degradation rates but also to identify significant metabolites in soil, these studies may have been inadequate to evaluate the full effect the storage of soil has on the route and rate of CPP degradation in soil.

The purpose of this laboratory study was to investigate how storage affects both the microbial community and the degradation rate and metabolism of metsulfuron-methyl [methyl 2-(4-methoxy-6-methyl-1,3,5-triazinyl-carbamoyl-sulfamoyl)benzoate]. The phospholipids fatty acid (PLFA) technique was used to monitor changes in a soil's microbial community (*20*). Metsulfuron-methyl was chosen due to its moderate soil half-life of 17−54 days (*21*−*24*), susceptibility to biological degradation (*22*−*27*), and complex pathway of degradation in soil, with several metabolites forming only through biotic degradation (*22*, *24*). Abiotic degradation of metsufluron-methyl was minimized in this study through the use of alkaline soils (pH $\geq$7.8) that were within the pH range at which metsulfuron-methyl was hydrolytically stable (*23*, *28*).

MATERIALS AND METHODS

Soil. Selected soil properties are listed in **Table 1**. The Trevino soil taxonomic classification was loamy, mixed, superactive, mesic Lithic Xeric Haplocambids, whereas the Fargo soil taxonomic classification was fine, smectitic, frigid Typic Epiaquerts. Soils were collected on October 8, 1998 (Trevino), and November 3, 1998 (Fargo). The top layer of vegetation from each soil was removed and the top 20 cm collected over several locations. Soils were composted and sieved moist through a 2 mm sieve prior to storage and stored in the dark at field moisture levels (50−60% of 33 kPa). The storage conditions tested in this study were the following: (1) fresh soil (stored for <3 weeks at 4 °C); (2) soil stored for 3 months at 4 °C; (3) soil stored for 3 months at 20 °C; (4) soil stored for 6 months at 4 °C; and (5) soil stored for 6 months at 20 °C. The microbial biomass and metsulfuron-methyl degradation were monitored for each soil storage treatment. In addition, the soil microbial biomass was monitored in the fresh soils that were incubated (in the dark at 20 °C) in the flow-through apparatus to test the effect the flow-through apparatus had on the microbial biomass. Soil moisture in the test vessels was checked periodically (approximately every 2 weeks) for loss of moisture and replenished with filtered sterile deionized water.

Chemicals. Non-radiolabeled metsulfuron-methyl and metabolite standards, 2[[[4-methoxy-6-methyl-1,3,5-triazin-2-yl-amino]carbonyl]-sulfonyl]benzoate (degradate **2**), methyl 2-[[(4-hydroxy-6-methyl-1,3,5-triazin-2-yl-amino]carbonyl]benzoate (degradate **3**), and methyl 2-(aminosulfonyl)benzoate (degradate **4**), 1,2-benzisothiazol-3(2*H*)-one (degradate **5**), were synthesized by DuPont Crop Protection, USA. See **Figure 1** for structures of metsulfuron-methyl and its metabolites. Radiolabeled [*phenyl*-U-^{14}C]metsulfuron-methyl was synthesized by NEN (Boston, MA) and had a radiochemical purity of >95% with a specific activity of 1.43 MBq mg^{-1} of metsulfuron-methyl. All organic solvents used were of HPLC grade or higher.

Soil Microbial Community Analysis. *Extraction and Purification*. Soil microbial biomass and community structure were determined by fatty acid methyl esters (FAME) analysis following the modified procedures of Findlay (*20*). At predetermined time intervals, soil samples were removed for FAME analysis. Three 10 g samples of soil were placed into 250 mL glass centrifuge bottles and extracted for 1 h with 50 mL of a methanol/chloroform/phosphate buffer (pH 7) solution (2:1:0.8 v/v/v) on a wrist action shaker. The extracted samples were centrifuged for 10 min at 2000 rpm and supernatants decanted (repeated twice). Pooled supernatants were placed into a 250 mL glass separatory funnel and washed with 50 mL of 2 M phosphate buffer (pH 7). The chloroform layer was removed and the aqueous layer extracted twice with 25 mL of chloroform. The pooled chloroform extracts (lipid extract) were dried with anhydrous magnesium sulfate and concentrated to ~1−2 mL using a Rapid Vap concentrator (Labconco Corp., Kansas City, MO). The lipid extracts were loaded onto a preconditioned silica gel solid phase extraction (SPE) column (500 mg, J. T. Baker, Phillipsburg, NJ) and separated into neutral and glyco- and phospholipids using sequentially 5 mL of chloroform, acetone, and methanol, respectively.

The phospholipid fraction (methanol extract) was evaporated to dryness under a gentle stream of nitrogen, redissolved into 0.5 mL of methanol/toluene (1:1 v/v), and subjected to a mild alkaline methanolysis with the addition of 0.2 M KOH in methanol (37 °C for 15 min). The resulting FAMEs were extracted three times with 2 mL of chloroform. The pooled extracts had 5 μL of an internal standard added (arachidic acid ethyl ester, Sigma-Aldrich, St. Louis, MO) and were gently reduced to near dryness under nitrogen. The FAME was purified by redissolving into acetonitrile and loading onto a preconditioned (hexane and chloroform) glass 500 mg C_{18} SPE column (J. T. Baker). The FAMEs were eluted from the column with 0.5 mL of hexane/chloroform (1:1 v/v). The hexane/chloroform fraction was gently brought to dryness under nitrogen and redissolved in 250 μL of hexane.

FAME Analysis. The purified FAME extracts were analyzed on a 6890 GC (Agilent Technologies, Inc., Wilmington, DE) equipped with a DB-5ms (30 m × 0.25 mm, 0.25 μm thickness) column (J&W Scientific, Wilmington, DE) and analyzed with either FID or mass spectrometer (MSD). The instrument was operated in constant flow mode at 1.5 mL min^{-1} and with a temperature program that increased from 110 to 300 °C at 15 °C min^{-1} and was held for 15 min at 300 °C. Gases used included helium (carrier and makeup) and oxygen and hydrogen for the combustion chamber of the FID. The FAMEs were identified by retention times from reference standards and library matches of mass spectra. Quantitation of individual FAMEs followed procedures described in Findlay (*20*).

Metsulfuron-nethyl Soil Metabolism. *Flow-through System.* The aerobic soil metabolism of [*phenyl*-(U)-^{14}C]metsulfuron-methyl was conducted in a flow-through apparatus consisting of 10 250-mL polypropylene centrifuge bottles containing 50 g (dry weight) of soil each. Each test vessel was fitted with an air inlet and outlet tubing that were connected to a series of four traps. The inlet of the flow-through apparatus was connected to a water bottle to provide humidified air. The outlet traps in the flow-through apparatus were in the following order: (1) blank overflow trap; (2) 2 M KOH (50 mL) trap, collected [^{14}C]CO_2 evolved; (3) 2 M KOH (50 mL) trap was used as a back-up to the first KOH trap; and (4) blank overflow trap. The outlet of the

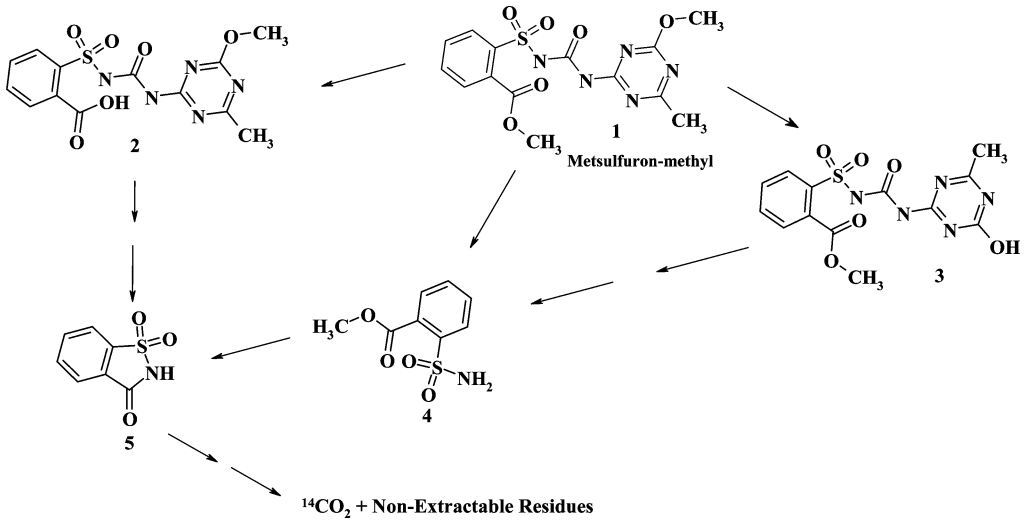

Figure 1. Chemical structures of metsulfuron-methyl and metsulfuron-methyl's degradates and proposed degradation pathway of metsulfuron-methyl in two alkaline soils.

last trap was connected to a vacuum manifold that was used to control the air flow in the whole test system. The apparatus was incubated in the dark at 20 °C during the course of the study. Two replicates per treatment were prepared.

Metsulfuron-methyl Application. Each test vessel was treated with 10 μg of [^{14}C]metsulfuron-methyl for a total of 14.3 kBq per vessel, which corresponds to a final metsulfuron-methyl concentration of 0.2 μg g^{-1} of oven dry weight soil or ~10 times field use rate. Metsulfuron-methyl was applied by pipet in an aqueous solution to bring the soil moisture contents to 75% of 33 kPa. Soils were mixed by hand with a specula for 2 min following the application of the test compound.

Soil Extraction. Soils were extracted for 1 h on a wrist action shaker with 100 mL of a saturated acetonitrile/2 M ammonium carbonate solution. The sample was centrifuged (10 min at 3000 rpm), the supernatant decanted, and the volume recorded. An aliquot (1 mL) of the supernatant was removed for radioactivity determination by liquid scintillation analysis (LSA) using a model 2100 TR (Packard BioScience Co., Meriden, CT). This process was repeated twice more (total of three extractions) with the pooled extracts being concentrated on a Turbo-Vap (Zymark Corp., Hopkinton, MA) to ~1−5 mL. Soil extracts were concentrated further under a gentle stream of nitrogen, transferred to 5 mL volumetric flasks, and diluted with 2 M ammonium carbonate buffer. Prior to HPLC analysis, extracts were filtered (>0.2 μm) centrifuged (15000 rpm, 5 min).

Nonextractable Residues. Extracted soils were air-dried in a laboratory hood for at least 1 week. Dried soils were homogenized and weighed, and triplicate aliquots (~0.5 g) were combusted (model OX-700 biological oxidizer, R. J. Harvey Instrument Corp., Hillsdale, NJ); the [^{14}C]CO_2 released from the combustion was trapped in a cocktail for LSA.

Volatile Radioactivity Analysis. At each sampling, trap solutions were removed and replaced with fresh trapping solutions. Trapping solution volumes were recorded and triplicate aliquots (1 mL) removed for LSA for $^{14}CO_2$ determination.

Analysis of Soil Extracts. Soil extracts were analyzed using an Agilent 1100 HPLC (Agilent Technologies, Inc.) equipped with both a photodiode array and in-line radiochemical detector (Ramona, Raytest, Inc., Wilmington, NC) and a fraction collector. The HPLC system separated radiolabeled compounds using a 4.6 mm × 250 mm RX-C8 Zorbax ODS HPLC column, a column flow of 1.5 mL min^{-1}, and a column temperature of 40 °C. A nonlinear gradient with a mobile phase of A (0.1% aqueous formic acid buffer) and B (acetonitile) used the following gradient: (1) 0−5 min, 100% A + 0% B; (2) 15 min, 85% A + 15% B; (3) 25 min, 60% A + 40% B; (4) 30 min, 0% A + 100% B; and (5) 30−35 min, 0% A + 100% B. The HPLC eluates were collected at 0.5 min intervals for 30 min and mixed with liquid scintillation cocktail for LSA. Radiochromatograms from fraction collection/LSA were reconstructed using an Excel-based program. Radiolabeled metsulfuron-methyl and its metabolites were identified by comparing chromatographic peak retention times to those of nonlabeled reference standards chromatographed and analyzed under the same conditions (taking into account the difference of ~0.3 min in elution time between detectors in series).

Statistical Analysis. *Soil Microbial Community Analysis.* Analysis of variance and mean separation (LSD) techniques were used to test for significant differences ($P < 0.05$) of the total FAME contents, as well as of the signature FAME representing either fungal, bacterial, or bacterial subgroup actinomycete populations. For each soil, storage treatments were analyzed by canonical discriminant analysis. Stepwise discriminant analysis was performed using PROC.STEPDISC from SAS to select the FAME most useful in distinguishing among the various soil treatments.

Metsulfuron-methyl Degradation. The natural log transformation data for metsulfuron-methyl degradation (loss of parent compound) versus time were fitted to a linear regression line. The half-lives and rates of mineralization were calculated from the fitted equations. Significant differences ($P < 0.05$) in the degradation and mineralization rates of metsulfuron-methyl were tested using analysis of variance of pooled means for the different storage treatments.

All statistical analyses were performed using either Statistical Analysis Systems version 8.2 (SAS Institute, Inc., Cary, NC) or JMP version 5.1 (SAS Institute, Inc.) statistical software.

RESULTS

The Trevino and Fargo soils were chosen for this study due to their different origins, climatic conditions, physical−chemical properties, and cropping systems; however, both soils did share similar soil pH values. The Trevino soil (American Falls, ID) was well drained, under potato cultivation, and located in a semiarid climate with a total annual precipitation in 1998 of 201 mm (*29*, *30*), whereas the Fargo soil (Grand Forks, ND) was poorly drained, under small grain cultivation, and located in cool subhumid climate with a total annual precipitation of 417 mm in 1998 (*29*, *30*). The Fargo soil clays were dominated by smectitic material (*29*).

Soil Microbial Community. *Trevino Soil.* Storage duration and temperature had a significant ($P < 0.05$) effect on microbial biomass (**Figure 2**). In fact, fresh soil biomass was significantly ($P < 0.05$) greater than that of soils stored for either 3 or 6 months. The temperature of storage also had a significant impact on the biomass, with soils stored at 4 °C retaining greater biomass than soils stored at 20 °C ($P < 0.001$), whereas there was no effect on biomass for soils stored for between 3 and 6

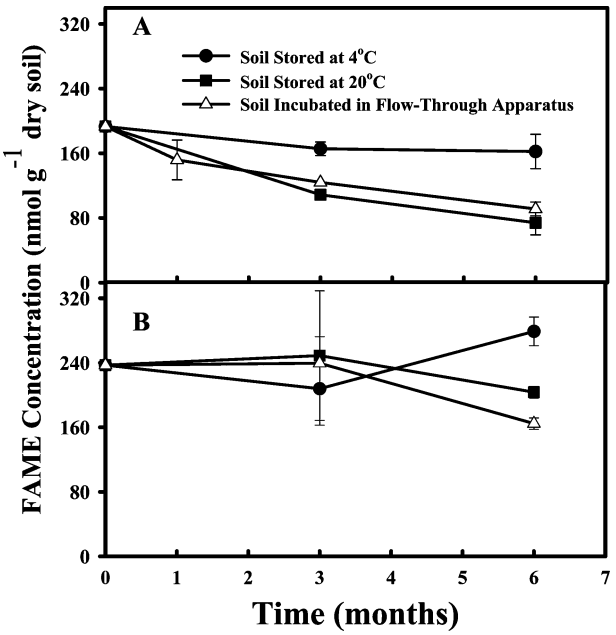

Figure 2. Changes in the concentration of FAME in soil either stored or incubated in a flow-through apparatus: (**A**) Trevino soil; (**B**) Fargo soil. Error bars represent the standard deviation of the mean.

Table 2. Phospholipid Fatty Acids Used as Biomarkers and Those Determined To Be Significant by Discriminate Analysis

indicator for[a]	fatty acid methyl ester[b]
Trevino Soil	
predominantly eubacteria	iC16:0, brC19, C16ω9t
microeukaryotes	C23:0, C22:1ω9, C22:2ω5
nondescriptive, widespread	C18:0, C16:1ω, C18:1ω, C19:1ω, 2-OH C14:0, 2-OH C16:0, 2-OH C20:0, 2-OH C22:0
Fargo Soil	
predominantly eubacteria	iC14:0, brC15:0, cyC17
microeukaryotes	C20:0, C20:1ω9, C20:5ω3
nondescriptive, widespread	C13:0, C14:0, C16:1ω, C18:1ω9t, 2-OH C12:0, 2-OH C16:0

[a] References *60* and *61*. [b] Fatty acids are designated as total number of carbon atoms:number of double bonds, with the position closest to the carboxyl (ω) end indicated and a "c" for cis or "t" for trans. The prefixes "i," "a," "cy," "Me", and "br" refer to iso, anteiso, cyclopropyl, methyl branching, and unknown methyl branching, respectively.

months. Trevino soils maintained in the flow-through apparatus had a declining biomass after only 28 days of incubation and declined to <50% of their initial content after only 6 months of incubation (**Figure 2**). In fact, there was no significant difference in the microbial biomass between soils stored at 20 °C and soils incubated in the flow-through apparatus at 20 °C.

The FAME associated with fungal biomass (**Table 2**) as a percent of the total microbial biomass in stored soils declined as storage temperature and storage duration increased (**Figure 3C**), with storage temperature having a greater impact on the fungal biomarker decline than storage duration. The percent fungal biomarker in the stored soils declined from 3.5 to 2.4%, whereas that of the soils incubated in the flow-through apparatus declined from 3.5 to 1.6% (**Figure 3C**). The FAME associated with bacterial biomarkers as a percent of the total microbial biomass in both the stored soils and the soils incubated in the flow-through apparatus followed no trends (**Figure 3A**), whereas the bacterial subgroup actinomycete biomarker increased with time in both the stored soils and the soils incubated in the flow-through apparatus (**Figure 3E**).

Fargo Soil. In general, stored Fargo soils behaved differently from the Trevino soil because storage of the Fargo soil did not significantly affect its total microbial biomass (**Figure 2**). However, the microbial biomass in the Fargo soils stored for 6 months at 20 °C did decline significantly ($P < 0.05$) compared to that of fresh soils. The Fargo soils incubated in the flow-through system followed trends similar to the Trevino soil with a significant ($P < 0.05$) decline in the total microbial biomass over time, but the decline was only ~30% after 6 months (**Figure 2**) compared to >50% in the Trevino soil. The percent fungal, bacterial, and actinomycete biomarkers to the total microbial biomass followed trends similar to those observed in the Trevino soil; that is, fungal biomarkers decreased with time, bacteria biomarkers followed no pattern, and actinomycete biomarkers increased with time (**Figure 3B**,**D**,**F**).

Discriminant Analysis (DA). The Trevino soil FAME profile demonstrates there were significant differences among all storage treatments (**Figure 4**). Temperature of storage had a greater impact on the microbial community than length of storage. The FAMEs that discriminated the different storage treatments are listed in **Table 2**. Three of the 14 FAME biomarkers were associated with fungi (**Table 2**) and 3 were associated with bacteria. The rest of the FAMEs were widespread or not associated with any particular group. The Fargo soil FAME profile had distinct groupings of soils according to storage treatment. There were no treatment effects associated with fresh soils or with soils stored for 3 months, whereas there were significant differences between fresh soils and soils stored for 6 months (**Figure 4**). In the case of Fargo soil, the length of storage had the greatest impact on the microbial community. The FAMEs that discriminated the different storage treatments are listed in **Table 2**. Three of the 12 FAMEs were associated with microeukaryote organisms, and 3 were associated with bacteria.

Only 2 of the 26 FAMEs that were used to discriminate between storage treatments for the two soils were common to both soils (**Table 2**). The FAMEs, 2-OH C16:0, 2-OH C20:0, 2-OH C22:0, and C22:1ω9 in the Trevino soil and 2-OH C12:0, 2-OH C16:0, and C13:0 in the Fargo soil, were identified from DA as the FAMEs partially responsible for differences in the microbial community between storage treatments. However, these FAMEs were actually only detected in samples later in the study. Similarly, Schutter and Dick (*31*) also found that the timing of when a particular FAME was detected had profound implications for the significance of that FAME in their multivariate analysis. They speculated that autoxidation of some FAMEs was occurring during soil storage and that this had the potential to drive changes in the FAME profile (*31*). Therefore, caution should be applied in the analysis of FAME patterns with multivariate analysis because small changes in certain FAMEs may skew the microbial community analysis.

Metsulfuron-methyl Degradation. *Trevino Soil.* The material balance in the Trevino soil study was excellent, with an average recovery of 101.1 ± 6.4% applied radioactivity (AR), and individual samples ranged from 84.3 to 114.5% AR (**Figure 5**). Degradation of metsulfuron-methyl in the Trevino soil appeared to follow pseudo-first-order kinetics because r^2 values ranged from 0.888 to 0.977 (**Table 2**). The degradation of metsulfuron-methyl was significantly faster ($P < 0.05$) in the fresh soil compared to stored soils (**Table 3**). Because there were no treatment effects in the degradation rate of metsulfuron-methyl among the different stored Trevino soils, the means of

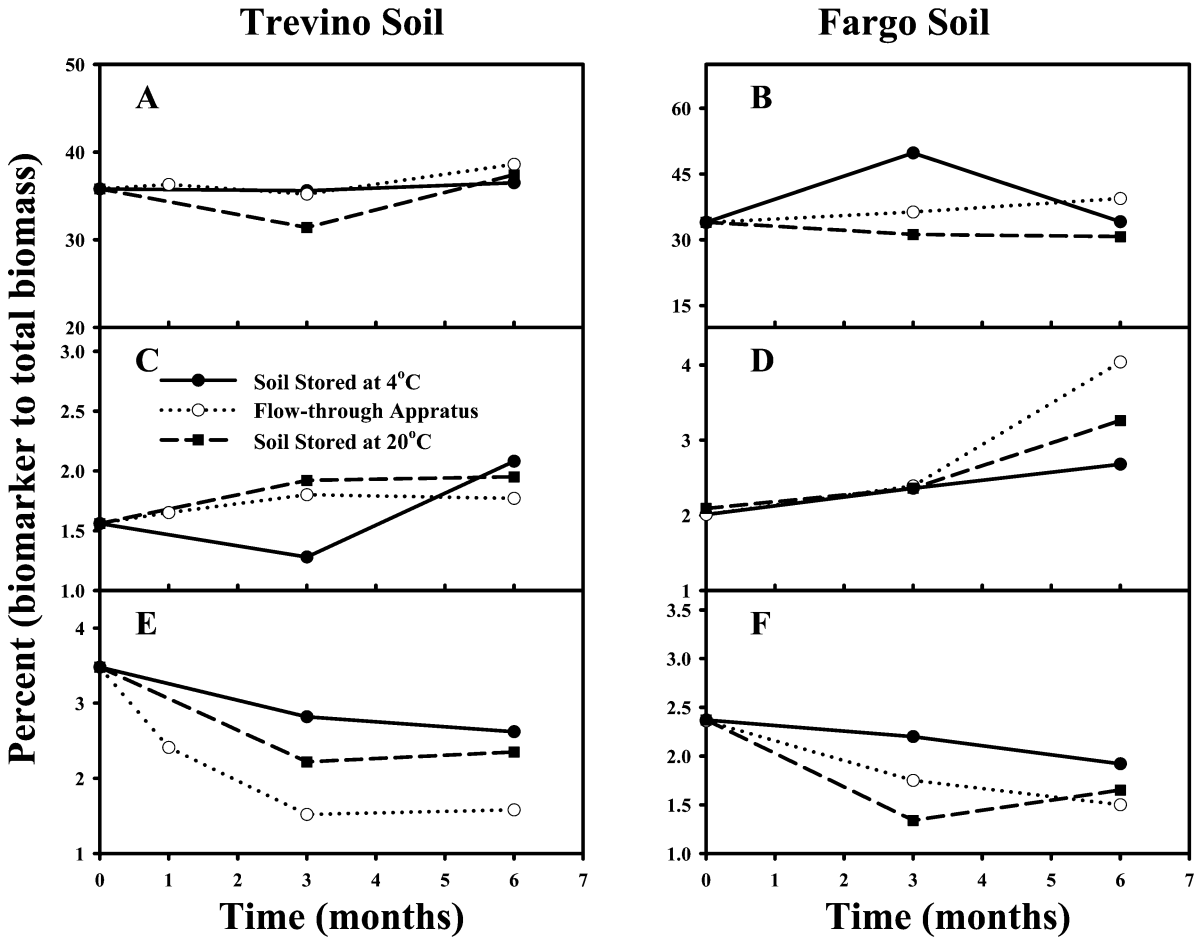

Figure 3. Changes in the percent biomarkers to total biomass for stored soils and soils incubated in a flow-through apparatus: (**A**, **B**) bacterial biomarker; (**C**, **D**) actinomycete biomarker; (**E**, **F**) fungal biomarker.

the stored soil's half-lives were pooled. Overall, the half-life of metsulfuron-methyl increased from 45 days (fresh soil) to >63 days for stored soils, which corresponds to a decrease in the degradation rate of 28%.

The mineralization of metsulfuron-methyl was significantly higher ($P < 0.05$) in the fresh soil compared to stored soils (**Figure 6**). Because there were no treatment effects among the stored Trevino soils in their mineralization of the phenyl ring of metsulfuron-methyl, means of the [^{14}C]CO_2 evolved for stored soils were pooled. Mineralization levels for fresh soils were 47.9% AR, whereas the mineralization levels in stored soils were 36.3% AR (24% reduction in amount mineralized). On the basis of degradation and mineralization results, storage of the Trevino soil affected metsulfuron-methyl degradation by 25%, with most of this change occurring in the first 3 months of storage.

Fargo Soil. The material balance in the Fargo soil study was excellent, with an average recovery of 102.0 ± 6.3% AR, and individual samples ranged from 84.1 to 118.3% AR (**Figure 5**). Degradation of metsulfuron-methyl in the Fargo soil did not follow simple first-order kinetics because r^2 values on average were <0.700 (**Table 2**). The metsulfuron-methyl degradation plots appeared to be biphasic (**Figure 5**); consequently, the degradation rate of metsulfuron-methyl was calculated using two pseudo-first-order equations. The first degradation rate was calculated for the first 28 days of the study, whereas the second degradation rate was calculated from day 28 to the termination of the study (day 119). The degradation of metsulfuron-methyl in the first 28 days appeared to follow pseudo-first-order kinetics, with r^2 values ranging from 0.744 to 0.848 (**Table 2**), but days 28−119 still resulted in a poor fit of the data with pseudo-first-order kinetics (r^2 ranged from 0.344 to 0.759). It should be noted that the poor fit of the data for days 28−119 to first-order kinetics is likely a result of little dissipation of metsulfuron-methyl occurring over that time period.

The temperature of storage had no effect on the degradation rate of metsulfuron-methyl in Fargo soil; as a result, means of different storage temperatures were pooled. The degradation rate of metsulfuron-methyl in the first 28 days of the study was significantly faster in the Fargo soils used either fresh or stored for 3 months compared to soils stored for 6 months ($P < 0.001$), whereas there were no treatment effects between fresh soils and soil stored for 3 months (**Table 3**). The half-life of metsulfuron-methyl in the Fargo soil increased from 23 days (fresh soil) to 29 days in soils stored for 6 months, which corresponds to a decrease in the degradation rate of 20%. There were no treatment effects in the degradation rate of metsulfuron-methyl between Fargo soils used fresh or stored (3 or 6 months) for the second half of the biphasic curve (days 28−119).

Mineralization of the phenyl ring of metsulfuron-methyl followed a trend similar to the mineralization in the Trevino soil. Namely, the mineralization was significantly higher ($P < 0.05$) in the fresh soil compared to stored soil (**Figure 6**). There were no significant differences in the mineralization of metsulfuron-methyl among stored soils. Mineralization levels in the fresh soils were 20.5% AR, whereas the mineralization levels in stored soils were 8.0% AR (60% reduction). On the basis of degradation and mineralization results, the storage of the Fargo soil reduced metsulfuron-methyl degradation by 20−60%.

Degradation Pathway. The degradation pathway of [^{14}C]-metsulfuron-methyl under aerobic conditions in alkaline soil is

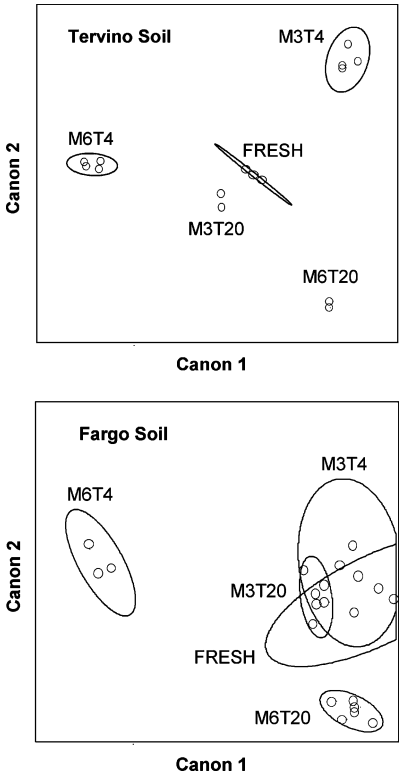

Figure 4. Score plots of the first two canonical functions showing separation of storage-temperature treatments by FAMEs for Trevino and Fargo soils: FRESH, <3 weeks of storage; M3T4, 3 months of storage at 4 °C; M3T20, 3 months of storage at 20 °C; M6T4, 6 months of storage at 4 °C; M6T20, 6 months of storage at 20 °C.

proposed in **Figure 1** and is similar to what was reported by Li et al. (*24*) for an acidic soil (pH 5.2). No degradation products were detected at >6% AR for any single time point (data not shown). Generally, the most noticeable difference in the degradation of metsulfuron-methyl in fresh soil compared to stored soil is loss of degradation product **2** in stored soils. Previously, this degradate was detected only in biologically active soil (*24*) and in our study was detected in both fresh soils and the Trevino soil stored for just 3 months. Detection of degradate **2** was typically early in the study (the first 28 days in fresh soils and the first 14 days for stored soils) with greater amounts of degradate **2** being detected in the fresh soils compared to stored soils.

DISCUSSION

Soil Microbial Community. Storage of the two soils affected their microbial biomass similarly in that a storage temperature of 20 °C had the most negative impact on the biomass for both soils. The Trevino soil's microbial biomass declined significantly after only 3 months of storage for both storage temperatures, and this decline was enhanced at elevated temperatures (20 °C), whereas the Fargo soil's microbial biomass started to decline only after 6 months of storage and at the elevated temperature of 20 °C. This is similar to what has been reported elsewhere in that storage temperatures of ∼5 °C or less tended to preserve the soil biomass over time (*7*, *10*, *12*, *42*), whereas storage temperatures of 20 °C or more tended to reduce the biomass more significantly (*7*, *10*, *12*). Overall, the Fargo soil's microbial community appeared to be more stable than the Trevino soil's as evidenced by a smaller decline in the total microbial biomass and less change to its FAME profile (**Figures 2** and **4**). The increased stability of the Fargo soil's microbial community in both the storage and flow-through apparatus experiments may be due in part to its high clay and organic matter contents compared to the Trevino soil (**Table 1**). Clay material can protect soil microorganisms from stress (*31*−*34*), improve storage of carbon substrate (*35*, *36*), and increase the diversity of a microbial community (*38*). In addition, soils with high clay contents have been shown to increase the survival of nonindigenous inoculated bacteria (*39*−*41*). Microbial communities of organic soils have been shown to be more stable than those of mineral soils during storage as measured by total biomass (*42*) and community structure (*31*). Given the decline in the Trevino soil microbial biomass, our results support EU guidelines of storing soils for <3 months at 4 °C (*4*). In fact, our data would suggest storing of some soils for even shorter periods of time, whereas other soils (i.e., soils with high clay and organic matter contents) may be suitable for longer periods of storage.

In addition, we investigated how the soil microbial biomass was affected by incubation in the flow-through apparatus, an experimental design recommended in regulatory guidelines for soil metabolism studies (*4*). Both soils that were incubated in the flow-through apparatuses showed significant declines in microbial biomass, despite maintenance of the water content in each soil during the study. As was the case in the soil storage study, the Trevino soil's microbial biomass had significant declines after only 3 months, whereas the Fargo soil's microbial biomass did not significantly decline until 6 months. Declines in the microbial biomass for soils incubated in the flow-through apparatus tracked closely to declines in the microbial biomass for the soil stored at 20 °C (**Figure 2**), including changes in the proportion of the fungal biomarker to total microbial biomass (**Figure 3**). These findings are consistent with those of others who have shown that during incubation the total microbial biomass declines with time (*43*) and that the proportion of fungal biomass to general biomass also declines (*42*, *43*). Nicolardot et al. (*44*) have shown that decomposition rates in soil decline by 60−70% after only 35−85 days of incubation at 20 °C. The general decline in the soil biomass after only 3 months of incubation confirms recommendations made by the EU to conduct soil metabolism studies for no longer than 120 days (*4*). Our results for some soils would actually caution against conducting a study for >90 days. The results in this study and those of Anderson (*7*) should strongly discourage the extension of soil metabolism studies past 120 days.

Degradation of Metsulfuron-methyl. The faster dissipation rate of parent compound in Fargo soil compared to Trevino soil was due in part to the amount and type of clay (smectite) associated with the Fargo soil because mineralization of metsulfuron-methyl was higher in Trevino soil compared to Fargo soil. Metsulfuron-methyl has been shown to be susceptible to degradation by smectite clays (*45*), which is shared by other sulfonylurea compounds (*46*−*48*). Heisterman et al. (*49*) have also shown that a significant portion of metsulfuron-methyl dissipation in soil is via kinetic sorption, and soils with high clay content have been shown to increase metsulfuron-methyl sorption (*50*, *51*). Despite differences in terms of physical−chemical properties and soil microbial community, both soils had significant declines in the degradation and mineralization of metsulfuron-methyl during soil storage.

Changes in the soil microbial community were not directly linked to changes in the degradation of metsulfuron-methyl. In the Trevino soil, the temperature of storage had a significant effect on both the size and structure of the microbial community, but had no significant effect on either the rate of metsulfuron-methyl degradation or its mineralization. Fargo soil's metsul-

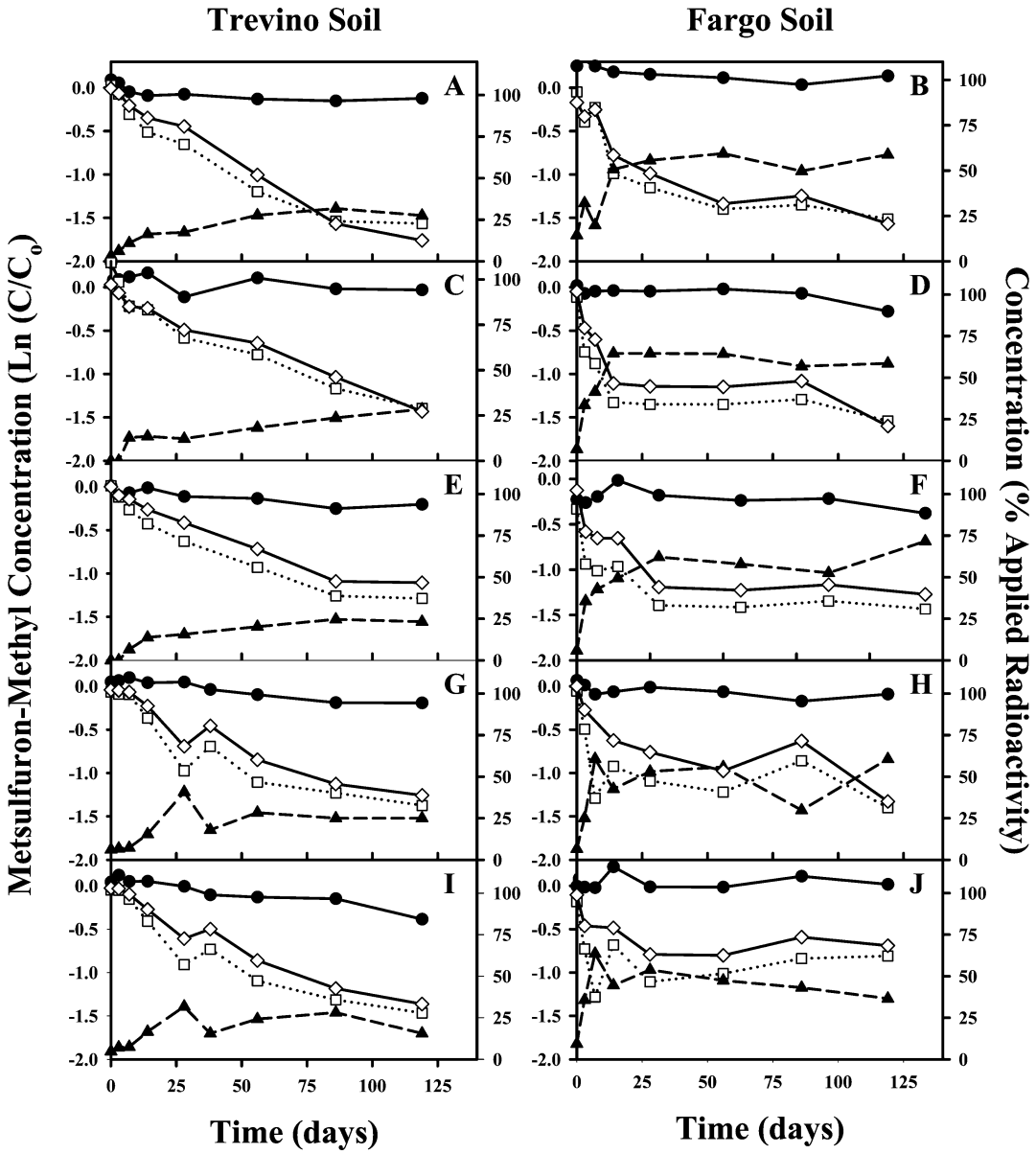

Figure 5. Total radioactivity (●), extractable radioactivity (□), nonextractable radioactivity (▲), and metsulfuron-methyl concentration (◇) in Trevino (first column) and Fargo (second column) soils: (**A**, **B**) fresh soil; (**C**, **D**) soil stored for 3 months at 4 °C; (**E**, **F**) soil stored for 3 months at 20 °C; (**G**, **H**) soil stored for 6 months at 4 °C; (**I**, **J**) soil stored for 6 months at 20 °C. The *Y* axis on the left is used for metsulfuron-methyl concentration, and the *Y* axis on the right is used for concentration in percent applied radioactivity.

furon-methyl degradation was loosely coupled with its soil microbial community because the duration of storage affected both the FAME profile and degradation rates. However, the size of the microbial community did not track with degradation trends of metsulfuron-methyl, nor did the mineralization of metsulfuron-methyl in Fargo soil track with any biomass trends. This lack of coupling between the soil microbial community and the degradation of metsulfuron-methyl is not surprising because attempts to correlate the degradation of other CPP with total microbial biomass have been unsuccessful (*52*), and soils with a history of CPP use degrading population sizes were estimated at $0.3-2800 \times 10^3$ degraders g^{-1} of soil or ~1−15% of the total microbial biomass (*53*−*55*). In this study, neither the Trevino nor the Fargo soils had any prior history of metsulfuron-methyl use; consequently, metsulfuron-methyl degrading populations were expected to be small and inconsequential compared to the total microbial biomass.

One of the unique results to come from our study is evidence of metsulfuron-methyl's pathway of degradation changing with soil storage. This was demonstrated by the lack of detection of degradation product **2** in both soils. This metabolite is one of the first transformation products of metsulfuron-methyl and has been previously detected in only biologically active soil (*24*). Loss of this metabolite suggests that part of the microbial community diversity was lost or inactivated during storage. Ramifications of this loss in a soil's functionality during storage would result in an incomplete regulatory evaluation of both the parent CPP and its soil metabolites. This emphasizes the importance of using fresh soils for the elucidation of degradation pathways of CPP in soil, which is emphasized in most regulatory test guidelines from U.S. and EU sources (*1*−*4*).

This paper appears to both contradict and agree with the previous work on the effect storage of soil has on the degradation of CPP (*7*, *18*, *19*). The current study shows that storage of soil does affect both its microbial biomass and its rate of degradation for CPP, similar to the findings of Anderson (*7*); however, metsulfuron-methyl degradation in soil was not correlated directly to changes in microbial biomass. The works

Table 3. Degradation Rates of Metsulfuron-methyl in Fresh and Stored Soils

storage duration (months)	storage temp (°C)	simple first order K_1 (10^{-2} day^{-1})	simple first order half-life[a] (days)	simple first order r^2	first-order biphasic K_1[b] (10^{-2} day^{-1})	first-order biphasic K_2[c] (10^{-2} day^{-1})	first-order biphasic half-life[d] (K_1') (days)	first-order biphasic half-life[e] (K_2') (days)	r_1^2	r_2^2
					Trevino Soil					
<3 weeks	4	1.54	45s	0.977	NA[f]	NA	NA	NA	NA	NA
storage pooled		1.10	63t	0.960	NA	NA	NA	NA	NA	NA
3	4	1.11	62t	0.955	NA	NA	NA	NA	NA	NA
3	20	0.98	71t	0.940	NA	NA	NA	NA	NA	NA
6	4	1.11	62t	0.888	NA	NA	NA	NA	NA	NA
6	20	1.24	56st	0.950	NA	NA	NA	NA	NA	NA
					Fargo Soil					
<3 weeks	4	1.11	63u	0.814	2.94	0.54	23w	128	0.848	0.759
3	pooled	0.81	86u	0.559	3.39	0.44	20w	158	0.744	0.301
3	4	0.85	81u	0.602	3.39	0.44	20w	158	0.744	0.301
3	20	0.74	93u	0.618	2.88	0.06	24w		0.832	
6	pooled	0.71	98v	0.622	2.36	0.48	29x	144	0.829	0.344
6	4	0.79	88v	0.668	2.31	0.46	30x	151	0.812	0.333
6	20	0.25	275v	0.240	1.76		39x		0.502	

[a] Within a soil, half-lives without a common letter differ, $P < 0.05$. [b] First-order rate constant generated for days 0–28. [c] First-order rate constant generated for days 28–119 soil. [d] Half-lives without a common letter differ, $P < 0.05$. [e] Half-lives without a common letter differ, $P < 0.05$ [f] Not applicable due to single first-order equation fitting of the data.

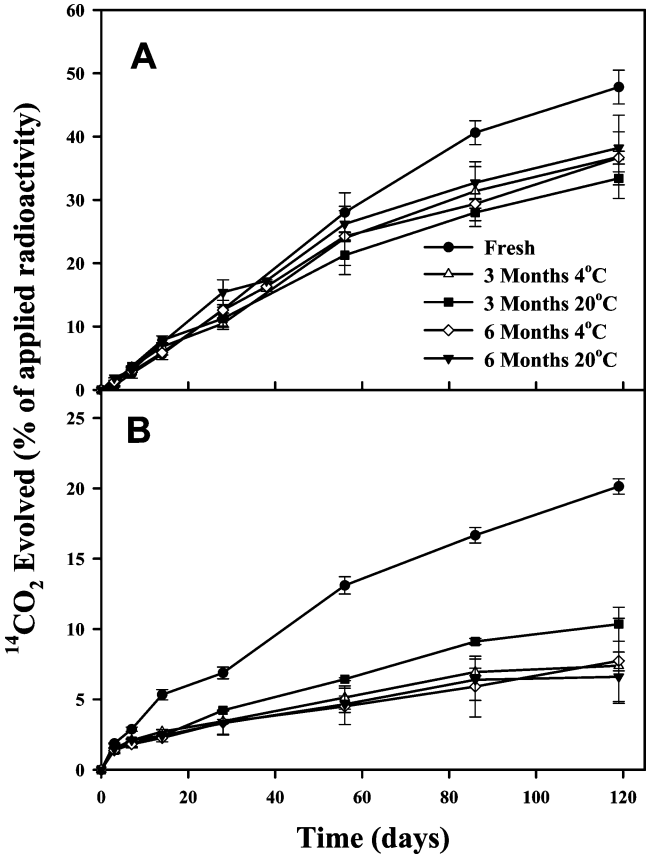

Figure 6. Mineralization of [^{14}C]metsulfuron-methyl in fresh soil or in soils stored for 3 or 6 months: (**A**) Trevino soil; (**B**) Fargo soil. Error bars represent the standard deviation of the mean.

of Stenberg et al. (*18*) and Posier et al. (*19*) showed that storing soils cold (4 °C) or frozen (−20 °C) had little effect on the degradation of CPP; however, our study shows that storage of soil significantly affects its capacity to degrade CPP. The discrepancies between our work and those of both Stenberg et al. (*18*) and Posier et al. (*19*) may be due to their experimental designs and the choice of CPP used to evaluate storage effects. In the Stenberg et al. (*18*) study, sample size (5 g) and replication number (single replicates) may have limited their abilities to detect significant changes in linuron's degradation rate. The technique they used to subdivide soil samples has been shown to be a source of variability in community-level physiological profiles (*56*), whereas the size of their samples (5 g) may have limited their ability to ensure homogeneity in sampling of their soil microbial community (*57*). In fact, Stenberg et al.'s (*18*) data do show a trend of slower degradation with increasing length of storage. In the Posier et al. (*19*) study, the detection of significant effects of storage on the degradation of methidathion may have been hindered by the short duration of the storage (4 days) and the inadequate sampling intervals (3, 7, and 14 days) used for measuring the mineralization rate of a compound with a half-life in soil of <8 days (*58*, *59*). Our study and that of Anderson (*7*) clearly show that storage of soil will affect the degradation of CPP susceptible to biological degradation.

Conclusion. Our study supports the current regulatory guideline recommendations for soil metabolism studies that the storage of soil should be kept to a minimum and that, if soils have to be stored, they should be stored for <3 months at 4 °C. We found that soil microbial biomass was significantly reduced after 3 months of storage and that storage at 4 °C preserved soil microbial biomass better than storage at 20 °C. However, we found no link between changes in soil microbial biomass during storage and changes in the degradation rate or route for metsulfuron-methyl in stored soil. This study shows that the storage of soil resulted in a reduction in the rate of metsulfuron-methyl degradation by 20−30% compared to fresh soil, whereas mineralization of metsulfuron-methyl was reduced by 25−60% during storage. We found no evidence that the temperature of storage had an effect on the degradation rate or mineralization of metsulfuron-methyl. Evidence for changes in the functional community during storage was demonstrated by an altered pathway of metsulfuron-methyl degradation in soil, and this change in the degradation pathway was similar for soils stored at either 4 or 20 °C. This study also supports the current regulatory guidelines of conducting soil metabolism for no longer than 120 days. We found that incubation of soil in a flow-through apparatus for >3 months resulted in significant losses (estimated to be 30−50%) in the soil microbial biomass.

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